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The *Acinetobacter baumannii* Biofilm-Associated Protein Plays a Role in Adherence to Human Epithelial Cells

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***Acinetobacter baumannii* is a significant source of nosocomial infections worldwide. This bacterium has the ability to survive and persist on multiple abiotic surfaces in health care facilities, and once a focus has been established, this opportunistic pathogen is difficult to eradicate. This paper demonstrates that the *A. baumannii* biofilm-associated protein (Bap) is necessary for mature biofilm formation on medically relevant surfaces, including polypropylene, polystyrene, and titanium. Scanning electron microscopy analyses of biofilms show that Bap is required for three-dimensional tower structure and water channel formation. In conjunction with persistence on abiotic surfaces, adherence to eukaryotic cells is an important step in bacterial colonization resulting in infection of the host. We have described Bap as the surface structure involved in adherence of *A. baumannii* to both normal human bronchial epithelial cells and normal human neonatal keratinocytes. However, Bap is not involved in internalization of the bacterium in these two cell lines. Furthermore, this study shows that the presence of Bap increases the bacterial cell surface hydrophobicity. The results of this study are pertinent, as the data lead to a better understanding of the role of Bap in biofilm formation on medical surfaces and in colonization of the host.**

Acinetobacter baumannii is a Gram-negative, aerobic, multidrug-resistant coccobaccillus that is of increasing importance in the medical community. *A. baumannii* is an opportunistic pathogen and a significant cause of nosocomial infections, although it is occasionally associated with community-acquired infections. While *A. baumannii* has been cultured from the skin of healthy individuals, it is more prevalent among patients in the hospital environment (1, 14). One of the main concerns with *A. baumannii* is its ability to persist in the hospital environment on various abiotic materials allowing contact with susceptible patients and causing outbreaks of ventilator-associated pneumonia, meningitis, bacteremia, and urinary tract and wound infections (1, 29). These infections are difficult to treat due to the emergence of multidrug-resistant strains (2, 18, 24, 34). These findings are an important indication that *A. baumannii* is a serious growing nosocomial threat worldwide.

While there have been studies defining important bacterial components expressed by *A. baumannii*, the mechanisms of virulence and persistence remain largely undefined. Those that have been described include the outer membrane protein A (OmpA) porin (6), K1 capsular polysaccharide (28), lipopolysaccharide (22), antimicrobial resistance genes (2, 18, 23, 24, 34), and plasmids containing organic peroxide resistance genes (13). Although little is known about the pathogenesis of disease caused by *A. baumannii*, the ability to persist in the environment on abiotic surfaces has been linked to biofilm formation (5, 11, 21, 31). In *A. baumannii*, the expression of *csuE*, which is part of the CsuA/BABCDE chaperone-usher assembly system of pili, is involved in the initial surface attachment during biofilm formation (12, 31). Poly-β-(1,6)-*N*-acetylglucosamine (PNAG) is an extracellular polysaccharide that is thought to function as an intercellular adhesin within the biofilm (5). The *blaPER-1* gene is also associated with increased cell adhesiveness and increased biofilm formation (19). Finally, the *A. baumannii* biofilm-associated protein (Bap) has been shown to play an important role in biofilm maturation and maintenance (21).

A. baumannii bap encodes a very large surface protein consisting of 8,621 amino acids that is homologous to the Bap protein first described in *Staphylococcus aureus* (8, 21). Production of Bap has been linked to initial adherence to abiotic surfaces, biofilm formation in both Gram-negative and Gram-positive bacteria, and to persistence and pathogenesis in the latter (8–10, 21, 26, 33). The *A. baumannii* Bap protein is essential for stabilization of mature biofilms on glass, affecting both thickness and biovolume (21). These data suggest that *A. baumannii* Bap is a key factor in biofilm formation and thus may have a role in persistence in the hospital environment and also in infection.

A previous study made the correlation that *A. baumannii* clinical isolates that adhere to epithelial cells at a high percentage also form large biofilm volumes (19). Although a number of bacterial factors have been implicated in biofilm formation, no specific surface structures have been defined for adherence. While there is very little known about the steps involved in *A. baumannii* pathogenesis, this opportunistic pathogen must initially adhere to host cell surfaces in order to cause disease. In this study, we determined that Bap expression affects cell surface hydrophobicity and biofilm formation on medically relevant surfaces. In addition, we present evidence for a role for Bap in adherence to relevant eukaryotic cells, suggesting that Bap may have an important role in colonization of the host.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. *A. baumannii* strain 307-0294 (*Ab*307) and the *A. baumannii* 307-0294 Bap-deficient mutant (*bap1302*::EZ-Tn*5* mutant) were described previously (21). Reverse transcription-PCR (RT-PCR) analysis of the gene (A1S_2695, hypothetical protein) immediately downstream of *bap* demonstrated that this gene was transcribed in the Bap mutant, suggesting that the Tn*5* disruption does not exert polar effects on neighboring genes (21). Bacteria were cultured in Mueller-Hinton (MH) medium at 37°C or on MH agar at 35.5°C and 5% CO_2 unless otherwise specified, with 50 μg/ml kanamycin supplementation as necessary.

Hydrophobicity. Cell surface hydrophobicity was assessed as described previously (17) with the following modifications. Briefly, cultures that had been grown on MH plates for 16 h were washed and resuspended in PUM buffer (22.2 g $K_2HPO_4 \cdot 3H_2O$, 7.26 g KH_2PO_4, 1.8 g urea, 0.2 g $MgSO_4 \cdot 7H_2O$; pH 7.1) to an optical density at 600 nm (OD_{600}) of 1.0. Part (1.6 ml) of each culture was incubated with 160 μl hexadecane in glass test tubes for 30 min at 35.5°C. Cultures were vortexed for 1 min and allowed to stand at room temperature for 15 min. The final OD_{600} of the aqueous culture was determined. Cell surface hydrophobicity was determined by calculating the BATH score (bacterial adhesion to hydrocarbon) as follows: $[1 - (OD_{final} - OD_{initial})] \times 100$ where OD_{final} is the final optical density and $OD_{initial}$ is the initial optical density. Three independent assays were performed with at least three replicates of each strain.

Biofilm assays. Biofilm formation on polystyrene, polypropylene, and titanium was assessed as described previously with minor modifications (32). Briefly, *Ab*307 and the *bap1302*::EZ-Tn*5* mutant were inoculated to an OD_{600} of 0.2 in Tris-M9 medium and were grown overnight at 37°C at 100 rpm. Biofilms were grown in polypropylene tubes in the presence of polystyrene slides or titanium rod sections. Materials containing biofilms were washed three times in phosphate-buffered saline (PBS) and stained with crystal violet. After washing with cold tap water, the biofilms were resuspended in 95% ethanol with the aid of glass beads and vortexing. The optical density at 595 nm was determined. Biofilms were grown in either duplicate or triplicate and were repeated at least three times.

SEM. Biofilms were developed as described above on polystyrene and titanium. Biofilms were washed in PBS and fixed for 1 h with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 0.075% ruthenium red and 0.075 M lysine acetate, pH 7.2. Samples were rinsed three times with 0.2 M sodium cacodylate buffer containing 0.075% ruthenium red (pH 7.2) and then subjected to graded incubations in 30%, 50%, 75%, 95%, and 100% ethanol. Samples were submerged twice in 100% hexamethyldisilazane and air dried. Scanning electron microscopy (SEM) images were captured with a Hitachi SU-70 or a Hitachi S-4000 microscope equipped with a tilt stage for side angle views.

Bacterial adherence assays. Bacterial adherence to eukaryotic cells was determined as previously described with some modifications (3, 25). Normal human bronchial epithelial (NHBE) and normal human neonatal keratinocyte (NHNK) primary cell lines, commercially available from Clonetics (Lonza Walkersville, Inc., Walkersville, MD) and InvitroCYTE (Seattle, WA), respectively, were cultured to confluence in 24-well tissue culture plates. *Ab*307 and the *bap1302*::EZ-Tn*5* mutant were cultured to mid-log phase and resuspended in eukaryotic cell culture medium. Eukaryotic cell monolayers were infected with 10^7 bacterial cells per well resulting in a multiplicity of infection (MOI) of 100:1. Plates were centrifuged at 220 $\times$ *g* for 5 min and incubated at 37°C with 5% CO_2 for 30 min, 90 min, 180 min, and 300 min. At each time point, eukaryotic monolayers were washed three times with HEPES-buffered saline solution (HBSS) (Clonetics) and harvested with trypsin. Harvested samples were dilution plated for enumeration of CFU following overnight incubation. Bacterial adherence was determined as the percentage of bacterial cells associated with eukaryotic cells relative to the original inoculum. A minimum of two independent assays was conducted, with each experimental condition performed in duplicate.

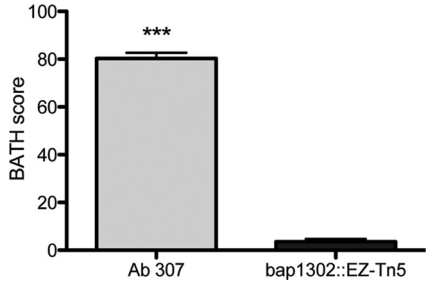

FIG 1 Cell surface hydrophobicity analyses of *A. baumannii* strain 307-0294 (*Ab*307) and the *bap1302*::EZ-Tn*5* mutant. The *bap1302*::EZ-Tn*5* mutant exhibits significantly decreased surface hydrophobicity compared to *Ab*307 when incubated with hexadecane. BATH score (bacterial adhesion to hydrocarbon) = $[1 - (OD_{final} - OD_{initial})] \times 100$ where OD_{final} is the final optical density and $OD_{initial}$ is the initial optical density. The values for *Ab*307 and the *bap1302*::EZ-Tn*5* mutant are significantly different ($P < 0.0001$) as indicated by the three asterisks. These data are presented as the means plus standard errors of the means (error bars) of 9 replicates of each strain.

Bacterial invasion assays. Bacterial invasion of NHBE and NHNK cells was determined as described previously with some modifications (7). The eukaryotic cells were cultured in 24-well tissue culture plates to confluence and infected as described above. At 180 min and 300 min, the monolayers were washed with HBSS, and fresh culture medium containing 300 μg/ml gentamicin sulfate was added to each well and incubated for 90 min. The monolayers were washed, harvested with trypsin, lysed with 1.0% saponin, and plated as described above. Bacterial invasion was calculated as the percentage of bacterial cells remaining after gentamicin sulfate treatment relative to cell-associated CFU. To determine whether internalization was active invasion by *Ab*307, NHNK and NHBE cells were preincubated 30 min with 1 μg/ml cytochalasin D (Sigma-Aldrich), and 1 μg/ml cytochalasin D was maintained in the media throughout the experiment. A minimum of two duplicate assays was conducted with each experimental condition performed in duplicate. To confirm that *bap* was transcribed under these conditions, RT-PCR was performed on RNA harvested from bacterial cells exposed to eukaryotic cell media using *bap*-specific primers 5′-TGCTGACAGTGACGTAGAACCACA-3′ and 5′-TGCAACTAGTGGAATAGCAGCCCA-3′. Pelleted bacterial cells exposed to eukaryotic cell media were dried on nitrocellulose membranes and probed with the Bap-specific monoclonal antibody MAb 6E3 to verify Bap expression (21).

Statistical analyses. Data are graphed as means $\pm$ standard errors of the means. Values were compared with the Student's *t* test using GraphPad Prism 5 software.

RESULTS

Bap contributes to cell surface hydrophobicity. It has been shown that organisms deficient in the expression of a Bap homolog have a decreased association with hydrocarbon (17, 30). This suggests that cell surface hydrophobicity may play a role in biofilm formation. To assess the contribution of *A. baumannii* Bap to cell surface hydrophobicity, *Ab*307 and the *bap1302*::EZ-Tn*5* mutant were incubated with the saturated hydrocarbon hexadecane. The Bap-deficient mutant (the *bap1302*::EZ-Tn*5* mutant) exhibited significantly decreased cell surface hydrophobicity in comparison to *Ab*307 ($P < 0.0001$), which readily incorporated into the hydrocarbon layer (Fig. 1).

Role of Bap in biofilm formation. The mechanisms utilized by *A. baumannii* to persist on abiotic surfaces remain undefined. The contribution of Bap was assessed by examination of biofilm formation on the medically relevant surfaces polystyrene, polypropylene, and titanium. Polystyrene and polypropylene were se-

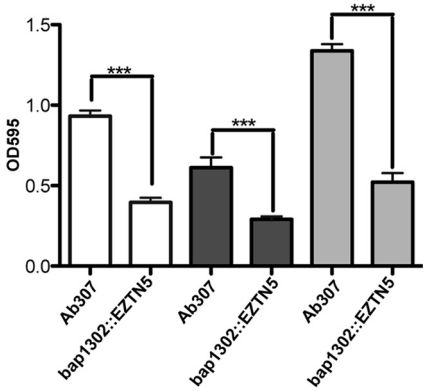

FIG 2 Comparisons of biofilm formation of *Ab*307 and the *bap1302*::EZ-Tn*5* mutant on medically relevant surfaces. *Ab*307 forms significantly more biofilm than the *bap1302*::EZ-Tn*5* mutant on three medically relevant materials; titanium (white), polystyrene (dark gray), and polypropylene (light gray). The values for *Ab*307 and the *bap1302*::EZ-Tn*5* mutant are significantly different ($P < 0.0001$) as indicated by the brackets and three asterisks. Solubilized crystal violet stained biofilms were quantitated by reading the absorbance at OD_{595}. These data are presented as the means plus standard errors of the means (error bars). Biofilms were cultured in duplicate or triplicate, and the assay was repeated at least three times.

lected for analysis because they are materials found routinely in a hospital setting, i.e., found in medical and nutritional packaging, test kit materials, parts used in pharmaceutical delivery systems, kitchen items, etc. Polypropylene is also used to produce nonabsorbable sutures and used in nonwoven medical textiles, whereas titanium was selected for study because it is a material often used in orthopedic procedures. Comparative biofilm studies showed that *Ab*307 forms significantly more biofilm than the Bap-deficient mutant on all three materials ($P < 0.0001$) (Fig. 2). Biofilms of *Ab*307 and the *bap1302*::EZ-Tn*5* mutant grown on polystyrene and titanium were imaged by scanning electron microscopy. Figure 3A shows that the wild-type *Ab*307 forms three-dimensional mature biofilm structures consisting of multicellular towers with obvious water channels between the towers on both surfaces. These towers are readily apparent in the side views shown in panels B and D, where the approximate height of these structures ranges between 55 and 82 μm. The Bap-deficient mutant (the *bap1302*::EZ-Tn*5* mutant) does not form a mature biofilm on either surface. While there appears to be minor differences in the number of bacteria attached to each surface, the side views clearly show that the mutant was incapable of forming multicellular structures, indicating that Bap is required for biofilm development and maturation on both polystyrene and titanium.

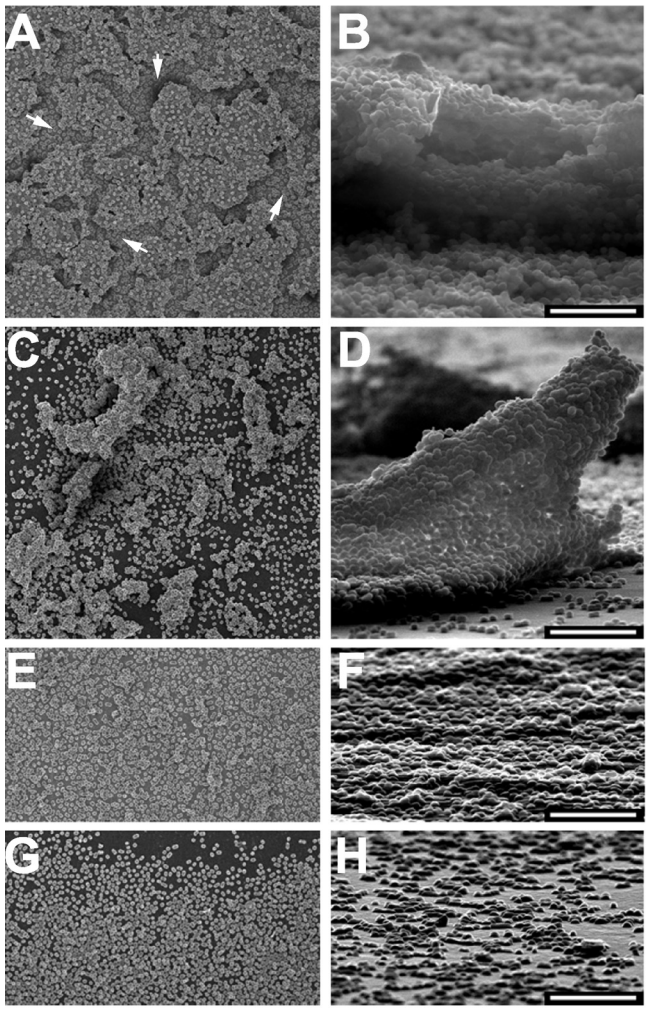

FIG 3 Phenotypic comparison of biofilm formation of *Ab*307 and the *bap1302*::EZ-Tn*5* mutant on titanium and polystyrene. SEM images were captured at a magnification of ×800 (left column) and from side tilt view at ×2,000 (right column). (A to D) *Ab*307 forms mature biofilm structures on titanium (A and B) and polystyrene (C and D) with classic towers and water channels (white arrows). The side views show examples of the complex multilayer structures that *Ab*307 formed in these biofilms with heights of approximately 55 μm on titanium (B) and 80 μm on polystyrene (D). (E to H) In contrast, the *bap1302*::EZ-Tn*5* mutant forms unorganized single layers of adherent cells on both titanium (E and F) and polystyrene (G and H). The side tilt view clearly shows that while the *bap1302*::EZ-Tn*5* mutant adheres to both surfaces, there are no multilayer structures or three-dimensional complex towers on either surface. White bars, 40 μm.

Bap expression is involved in adherence to human eukaryotic cells. To determine whether surface expression of Bap affects the association of *Ab*307 with eukaryotic cell surfaces, adherence assays were performed using primary NHBE and NHNK cells. NHBE cells were selected because *A. baumannii* is a causative agent of ventilator-associated pneumonia. Since *A. baumannii* is also associated with wound infections and has been shown to colonize the skin of health care workers who can unknowingly transfer *A. baumannii* to susceptible patients, NHNK cell adherence was also assessed. Monolayers were infected with 10^7 bacterial cells/well (MOI of 100:1) for 30 min, 90 min, 180 min, and 300 min. Figure 4 demonstrates that *Ab*307 associated with NHBE cells (Fig. 4A) and NHNK cells (Fig. 4B) at a significantly higher percentage than the *bap1302*::EZ-Tn*5* mutant ($P < 0.02$). The greatest difference in adherence occurred at 180 min where approximately 45% of *Ab*307 adhered to both NHBE and NHNK cells while less than 15% of the *bap1302*::EZ-Tn*5* mutant was adherent. Although both strains exhibited comparable growth at the 300-min incubation point, the level of *Ab*307 adherence to NHBE and NHNK remained 3-fold higher than that of the *bap1302*::EZ-Tn*5* mutant (data not shown). RT-PCR analysis using *bap*-specific internal primers and whole-cell immunoblots with the previously described Bap-specific antibody MAb 6E3 showed that *bap* is being transcribed and expressed by *Ab*307 under these assay conditions (data not shown) (21). These data indicate that Bap

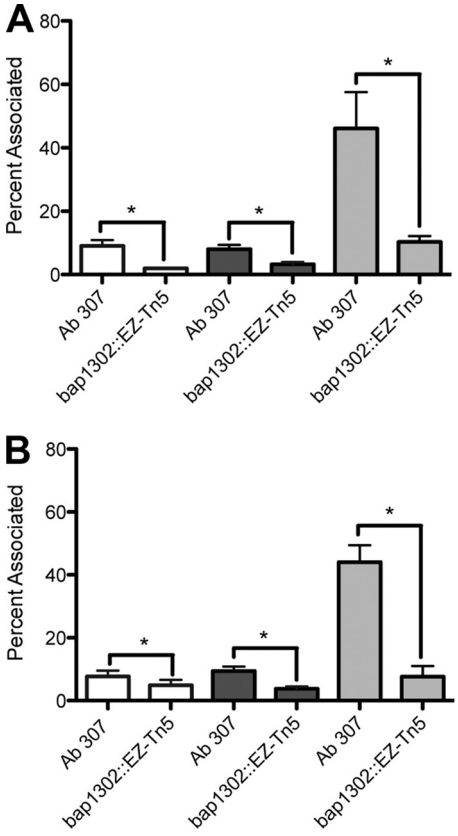

FIG 4 Bacterial association with NHBE and NHNK cells. (A and B) *Ab*307 adheres significantly more than the *bap1302*::EZ-Tn*5* mutant to NHBE (A) and NHNK cells (B) at 30 min, 90 min, and 180 min. The values for *Ab*307 and the *bap1302*::EZ-Tn*5* mutant are significantly different ($P < 0.02$) as indicated by the bracket and asterisk. Data are presented as the means plus standard errors of the means (error bars).

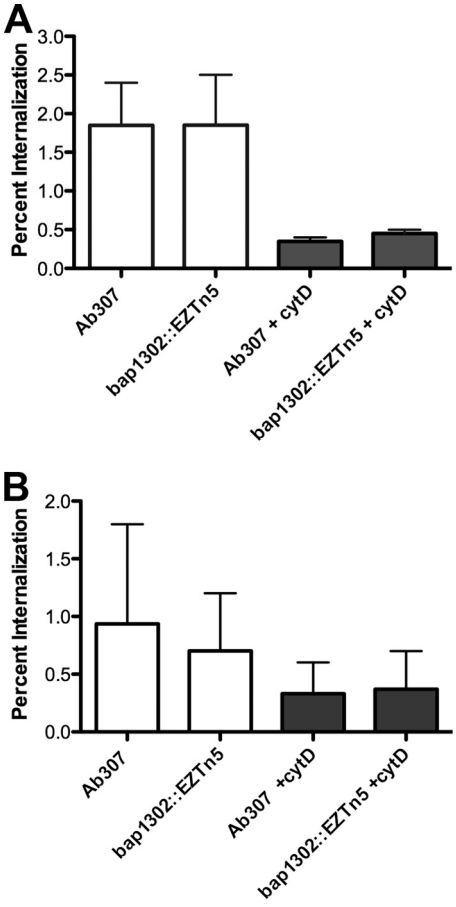

FIG 5 Internalization of *Ab*307 and the *bap1302*::EZ-Tn*5* mutant at 180 min. (A and B) There is no significant difference in the internalization of *Ab*307 in comparison to the *bap1302*::EZ-Tn*5* mutant by NHBE (A) or NHNK cells (B). The addition of 1 μg/ml cytochalasin D (cytD) (dark gray bars) reduces the quantity of bacteria internalized by the cells; however, these data are not significantly different. Data are presented as the means plus standard errors of the means (error bars).

functions as an adhesin involved in *A. baumannii* attachment to both NHBE and NHNK cells *in vitro*.

Bap expression does not play a role in NHBE or NHNK cell invasion. To determine whether expression of Bap affects the invasion of NHBE and NHNK cells by *Ab*307, gentamicin protection assays were performed. In these studies, we analyzed invasion after 180 min and 300 min of incubation, as these time points resulted in the maximal adherence levels. There was no significant difference between internalization of *Ab*307 and the *bap1302*::EZ-Tn*5* mutant by either NHNK or NHBE cells at 180 min (Fig. 5) or 300 min (data not shown). Approximately 2% of total adherent *Ab*307 and the *bap1302*::EZ-Tn*5* mutant were internalized at both time points. These data are consistent with a previous study demonstrating a low level of invasion for different strains of *A. baumannii* (7). These investigators also determined that incubation times exceeding 5 h resulted in decreased eukaryotic cell viability (7). In the presence of the actin polymerization inhibitor cytochalasin D, both strains were internalized to a lesser extent by each eukaryotic cell line, although these data were not statistically significant (Fig. 5). Cytochalasin D did not affect bacterial viability (data not shown). These data indicate that Bap is not involved in internalization of or invasion by *A. baumannii* in the cell lines examined.

DISCUSSION

The purpose of this study was to determine whether the *A. baumannii* Bap had an important role in cell surface hydrophobicity and biofilm formation on medically relevant surfaces and also to determine whether this protein was involved in eukaryotic cell adherence, an important step in host colonization.

In silico analysis of *A. baumannii* Bap indicated that the protein itself has a high degree of hydrophilicity (27). However, our data show that wild-type *Ab*307 cells expressing Bap readily incorporate into a hexadecane layer to a significantly greater degree than Bap-deficient cells with a BATH score 23-fold higher than the Bap mutant. Therefore, although the sequence was predicted to be hydrophilic by *in silico* modeling, the expression of Bap increases the overall cell surface hydrophobicity. Interestingly, *A. baumannii* Bap contains conserved sequences that are also found in the hyaline repeat (HYR) module (4, 21). The HYR module structure resembles an immunoglobulin-like fold and was first described to function in eukaryotic cell adhesion proteins and has also been found in bacterial adhesion proteins (4). The HYR domain also contains conserved hydrophobic residues (4). These conserved residues in Bap may contribute to the cell surface hydrophobicity.

Previous studies have shown that *Ab*307 Bap plays a role in static biofilm maturation and maintenance, increasing both biofilm thickness and biovolume on glass surfaces (21). To determine whether Bap expression is involved in biofilm formation on medically relevant surfaces, biofilm studies were performed on polystyrene, polypropylene, and titanium. Cells expressing Bap formed more biofilm on all three materials. It is important to note that these studies were performed with materials suspended in bacterial cultures under constant agitation. The results of SEM analyses of these bacteria on polystyrene and titanium indicated that Bap-expressing cells form the classic biofilm architecture consisting of multidimensional towers separated by water channels. Some of these tower structures were quite prominent in the biofilm, and they were fairly stable, as they remained intact despite constant agitation. In contrast, the Bap-deficient cells remained predominantly in a single layer with a mat-like phenotype containing a few sparse areas of cell aggregates. There were no obvious tower structures formed by this mutant. These data provided two important observations. First, while the Bap mutant did not form a mature biofilm, this construct is fully capable of attaching to different materials. Thus, *A. baumannii* Bap does not play a major role in the initial phases of adherence to abiotic surfaces. These results contrast with previous reports demonstrating that the *S. aureus* Bap is involved in initial attachment to surfaces, suggesting that these two homologs may have somewhat divergent functions (8). However, *A. baumannii* Bap is clearly required for biofilm development and maturation on both polystyrene and titanium. The mechanism behind this maturation is not fully understood. It is possible that Bap binds to other Bap molecules on neighboring cells, thus functioning as intercellular adhesins within the biofilm and contributing to the overall structural support and integrity of the developing biofilm. Another possibility is that Bap may bind another molecule within the biofilm. Interestingly, *in silico* analysis of the Bap sequence using InterProScan software (27) revealed that *Ab*307 Bap contains homologous domains to the PKD (polycystic kidney disease) domain and the concanavalin A-like lectin/glucanase domain. The PKD domain has been implicated in protein-protein interactions, whereas the presence of the concanavalin A-like lectin/glucanase domain suggests possible protein-carbohydrate interactions (27). Thus, the structural composition of *A. baumannii* Bap suggests that future studies should focus on possible protein-protein and protein-carbohydrate interactions.

Bap was detected on the surface of 43% (42/98) of the *A. baumannii* clinical isolates evaluated in a previous phenotypic analysis using the Bap-specific monoclonal antibody MAb 6E3 (21). These data demonstrate that Bap expression is fairly conserved among a diverse panel of isolates and not limited to a few strains. However, it should be noted that the nonreactive strains of *A. baumannii* may not be Bap deficient, but instead they may express a Bap that does not contain the specific epitope recognized by MAb 6E3. A BLAST search of the NCBI database demonstrates that a portion of the *Ab*307 *bap* is present in all *A. baumannii* genome sequences; however, the complete sequence of the coding region is not present in these other strains. This is likely due to the extensive number of repeats in *bap*, which also precludes the ability to perform accurate molecular analyses designed to identify the genetic level of conservation.

The biofilm data presented in this study confirm that Bap is an essential component in biofilm development and demonstrate that this surface protein is required in the formation of a classic biofilm phenotype on a variety of different surfaces, including those commonly found in health care facilities. These data are important because the ability of *A. baumannii* to survive and persist on abiotic materials is linked to outbreaks of nosocomial infections. In order to develop more effective ways of eradicating *A. baumannii* contamination from the environment, we must identify the bacterial factors that are important for persistence, and our data implicate Bap as a key component.

It has been previously determined that *A. baumannii* adheres to human bronchial epithelial cells (11, 16, 19, 20) and human alveolar epithelial cells (7, 15), although the mechanism of adherence was not defined. Our findings show that cells expressing Bap on the surface adhered to both NHBE and NHNK cells at significantly greater percentages than Bap-deficient cells at an early time point of 30 min with increasing adherence over time. These data are novel because they provide evidence suggesting that the *A. baumannii* Bap contributes to eukaryotic cell adherence. Another important aspect of our data is the fact that we have used primary human cell lines. While previous studies have provided valuable insight into *A. baumannii* adherence and invasion of multiple eukaryotic cell lines, these studies all used transformed cells, which likely differ from the primary human cells that *A. baumannii* encounters in the initial stages of colonization (7, 16, 19, 20).

In addition, Bap may be an important factor in adherence because of its propensity to increase the surface hydrophobicity of the bacteria. A previous study of the adherence of group B streptococci to epithelial cells showed that hydrophobic interactions between bacterial surface proteins and the epithelial cell surface is the mechanism for adherence (35). Our data support the hypothesis that Bap plays a role in eukaryotic cell adherence; however, we did not find evidence to support a role for Bap in internalization. There was no significant difference between *Ab*307 and the *bap1302*::EZ-Tn*5* mutant in internalization by either NHBE or NHNK cells. Previous work by Choi et al. determined that *A. baumannii* invasion of epithelial cells is dependent on OmpA and the action of eukaryotic cell microtubules and microfilaments (7). These authors also reported a fairly low level of invasion and concluded that both adherence and invasion are likely dependent on the specific *A. baumannii* strain and the eukaryotic cell line being evaluated. On the basis of those data, we would not expect to see differences in internalization between the Bap-expressing cells and the Bap-deficient cells. As expected, there were no significant differences in internalization between the mutant and wild type in either eukaryotic cell line tested. The results of our studies demonstrate that *Ab*307 does not actively internalize in these cell lines under the *in vitro* conditions we tested. Rather, our data suggest that internalization of the organism is dependent upon active uptake by the eukaryotic cells.

Unfortunately, the size of the Bap gene precludes complementation of the mutant, and this represents a limitation of our current study. However, in our initial characterization of the Bap mutant, we confirmed that transcription of the A1S_2695 gene, which codes for a hypothetical protein immediately downstream of *bap*, was not affected by the transposon insertion (21). In addition, we confirmed that *bap* and A1S_2695 were not cotranscribed. These data suggest that the *bap1302*::EZ-Tn*5* mutant is likely nonpolar, although we cannot rule out any undetectable effects in other regions of the chromosome.

In conclusion, our data demonstrate that Bap plays a key role in biofilm formation and maturation on abiotic surfaces of med-

ical importance. Also, Bap increases bacterial cell surface hydrophobicity, which may be a mechanism involved in the increased adherence of *Ab*307 to both NHBE and NHNK cells. Adherence to eukaryotic cells is an important step in colonization and host infection. Thus far, Bap is the only *A. baumannii* surface protein that has been linked to both biofilm formation on abiotic materials and attachment to primary human cells encountered in the host. Taken together, these data represent important contributions to the field of *A. baumannii* research. First, our data identify Bap as a potential target for future studies focused on the development of novel antimicrobial methods designed to eliminate *A. baumannii* from abiotic and medically relevant surfaces. Second, our data further implicate Bap as a potential target for novel therapeutic methods designed to prevent *A. baumannii* colonization and infection of the host. These are critical observations, because in order to control *A. baumannii* infections, it is essential to understand the mechanisms of environmental persistence and the mechanisms of colonization.

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REFERENCES

1. **Bergogne-Berezin E, Towner KJ.** 1996. *Acinetobacter* spp. as nosocomial pathogens: microbiological, clinical, and epidemiological features. Clin. Microbiol. Rev. **9**:148–165.
2. **Bonomo RA, Szabo D.** 2006. Mechanisms of multidrug resistance in *Acinetobacter* species and *Pseudomonas aeruginosa*. Clin. Infect. Dis. **43**(Suppl. 2):S49–S56.
3. **Brentjens RJ, Spinola SM, Campagnari AA.** 1994. *Haemophilus ducreyi* adheres to human keratinocytes. Microb. Pathog. **16**:243–247.
4. **Callebaut I, Gilges D, Vigon I, Mornon JP.** 2000. HYR, an extracellular module involved in cellular adhesion and related to the immunoglobulin-like fold. Protein Sci. **9**:1382–1390.
5. **Choi AH, Slamti L, Avci FY, Pier GB, Maira-Litran T.** 2009. The *pgaABCD* locus of *Acinetobacter baumannii* encodes the production of poly-beta-1-6-N-acetylglucosamine, which is critical for biofilm formation. J. Bacteriol. **191**:5953–5963.
6. **Choi CH, et al.** 2005. Outer membrane protein 38 of *Acinetobacter baumannii* localizes to the mitochondria and induces apoptosis of epithelial cells. Cell. Microbiol. **7**:1127–1138.
7. **Choi CH, Lee JS, Lee YC, Park TI, Lee JC.** 2008. *Acinetobacter baumannii* invades epithelial cells and outer membrane protein A mediates interactions with epithelial cells. BMC Microbiol. **8**:216.
8. **Cucarella C, et al.** 2001. Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. J. Bacteriol. **183**:2888–2896.
9. **Cucarella C, et al.** 2002. Expression of the biofilm-associated protein interferes with host protein receptors of *Staphylococcus aureus* and alters the infective process. Infect. Immun. **70**:3180–3186.
10. **Cucarella C, et al.** 2004. Role of biofilm-associated protein *bap* in the pathogenesis of bovine *Staphylococcus aureus*. Infect. Immun. **72**: 2177–2185.
11. **de Breij A, et al.** 2010. Do biofilm formation and interactions with human cells explain the clinical success of *Acinetobacter baumannii*? PLoS One **5**:e10732.
12. **de Breij A, et al.** 2009. CsuA/BABCDE-dependent pili are not involved in the adherence of *Acinetobacter baumannii* ATCC19606(T) to human airway epithelial cells and their inflammatory response. Res. Microbiol. **160**: 213–218.
13. **Dorsey CW, Tomaras AP, Actis LA.** 2006. Sequence and organization of pMAC, an *Acinetobacter baumannii* plasmid harboring genes involved in organic peroxide resistance. Plasmid **56**:112–123.
14. **Fournier PE, Richet H.** 2006. The epidemiology and control of *Acinetobacter baumannii* in health care facilities. Clin. Infect. Dis. **42**:692–699.
15. **Gaddy JA, Actis LA.** 2009. Regulation of *Acinetobacter baumannii* biofilm formation. Future Microbiol. **4**:273–278.
16. **Gaddy JA, Tomaras AP, Actis LA.** 2009. The *Acinetobacter baumannii* 19606 OmpA protein plays a role in biofilm formation on abiotic surfaces and in the interaction of this pathogen with eukaryotic cells. Infect. Immun. **77**:3150–3160.
17. **Huber B, et al.** 2002. Genetic analysis of functions involved in the late stages of biofilm development in *Burkholderia cepacia* H111. Mol. Microbiol. **46**:411–426.
18. **Hujer KM, et al.** 2006. Analysis of antibiotic resistance genes in multidrug-resistant *Acinetobacter* sp. isolates from military and civilian patients treated at the Walter Reed Army Medical Center. Antimicrob. Agents Chemother. **50**:4114–4123.
19. **Lee HW, et al.** 2008. Capacity of multidrug-resistant clinical isolates of *Acinetobacter baumannii* to form biofilm and adhere to epithelial cell surfaces. Clin. Microbiol. Infect. **14**:49–54.
20. **Lee JC, et al.** 2006. Adherence of *Acinetobacter baumannii* strains to human bronchial epithelial cells. Res. Microbiol. **157**:360–366.
21. **Loehfelm TW, Luke NR, Campagnari AA.** 2008. Identification and characterization of an *Acinetobacter baumannii* biofilm-associated protein. J. Bacteriol. **190**:1036–1044.
22. **Luke NR, et al.** 2010. Identification and characterization of a glycosyltransferase involved in *Acinetobacter baumannii* lipopolysaccharide core biosynthesis. Infect. Immun. **78**:2017–2023.
23. **Marchand I, Damier-Piolle L, Courvalin P, Lambert T.** 2004. Expression of the RND-type efflux pump AdeABC in *Acinetobacter baumannii* is regulated by the AdeRS two-component system. Antimicrob. Agents Chemother. **48**:3298–3304.
24. **Navon-Venezia S, Leavitt A, Carmeli Y.** 2007. High tigecycline resistance in multidrug-resistant *Acinetobacter baumannii*. J. Antimicrob. Chemother. **59**:772–774.
25. **Plamondon P, Luke NR, Campagnari AA.** 2007. Identification of a novel two-partner secretion locus in *Moraxella catarrhalis*. Infect. Immun. **75**: 2929–2936.
26. **Potter A, et al.** 2009. The gene *bap*, involved in biofilm production, is present in *Staphylococcus* spp. strains from nosocomial infections. J. Microbiol. **47**:319–326.
27. **Rahbar MR, Rasooli I, Mousavi Gargari SL, Amani J, Fattahian Y.** 2010. In silico analysis of antibody triggering biofilm associated protein in *Acinetobacter baumannii*. J. Theor. Biol. **266**:275–290.
28. **Russo TA, et al.** 2010. The K1 capsular polysaccharide of *Acinetobacter baumannii* strain 307-0294 is a major virulence factor. Infect. Immun. **78**:3993–4000.
29. **Sheppard FR, et al.** 2010. The majority of US combat casualty soft-tissue wounds are not infected or colonized upon arrival or during treatment at a continental US military medical facility. Am. J. Surg. **200**:489–495.
30. **Toledo-Arana A, et al.** 2001. The enterococcal surface protein, Esp, is involved in *Enterococcus faecalis* biofilm formation. Appl. Environ. Microbiol. **67**:4538–4545.
31. **Tomaras AP, Dorsey CW, Edelmann RE, Actis LA.** 2003. Attachment to and biofilm formation on abiotic surfaces by *Acinetobacter baumannii*: involvement of a novel chaperone-usher pili assembly system. Microbiology **149**:3473–3484.
32. **Tomaras AP, Flagler MJ, Dorsey CW, Gaddy JA, Actis LA.** 2008. Characterization of a two-component regulatory system from *Acinetobacter baumannii* that controls biofilm formation and cellular morphology. Microbiology **154**:3398–3409.
33. **Tormo MA, et al.** 2007. Phase-variable expression of the biofilm-associated protein (Bap) in *Staphylococcus aureus*. Microbiology **153**: 1702–1710.
34. **Tsakris A, Ikonomidis A, Pournaras S, Spanakis N, Markogiannakis A.** 2006. Carriage of OXA-58 but not of OXA-51 beta-lactamase gene correlates with carbapenem resistance in *Acinetobacter baumannii*. J. Antimicrob. Chemother. **58**:1097–1099.
35. **Wibawan IT, Lammler C, Pasaribu FH.** 1992. Role of hydrophobic surface proteins in mediating adherence of group B streptococci to epithelial cells. J. Gen. Microbiol. **138**:1237–1242.